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(71) Applicant *(for all designated States except US)*: **KOREA RESEARCH INSTITUTE OF BIOSCIENCE AND BIOTECHNOLOGY** [KR/KR]; 52 Oun-dong, Yusung-ku, Taejon-si 305-333 (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KWAK, Sang-Soo** [KR/KR]; #307-306 Expo Apt., 464-1 Jeonmin-dong, Yusung-ku, Taejon-si 305-390 (KR). **LEE, Haeng-Soon** [KR/KR]; #126-502 Hanbit Apt., 99 Oun-dong, Yusung-ku, Taejon-si 305-333 (KR). **KWON, Suk-Yoon** [KR/KR]; #119-902 Hanbit Apt., 99 Oun-dong, Yusung-ku, Taejon-si 305-333 (KR). **KIM, Chang-Jin** [KR/KR]; #7-203 Townhouse, 391 Kuryoung-dong, Yusung-ku, Taejon-si 305-504 (KR). **LEE, Hyang-Burm** [KR/KR]; #126-803 Hanbit Apt., 99 Oun-dong, Yusung-ku, Taejon-si 305-755 (KR). **LEE, Sang-Han** [KR/KR]; #2-505 Kwakiwon Apt., 383-2 Doryoung-dong, Yusung-ku, Taejon-si 305-340 (KR).

(74) Agent: **LEE, Won-Hee**; 8th Fl., Sung-ji Heights II, 642-16 Yoksam-dong, Kangnam-ku, Seoul 135-080 (KR).

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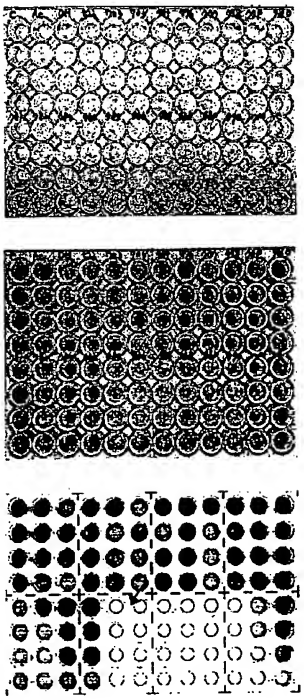

(57) Abstract: The present invention relates to a method for high throughput screening of plant growth regulator, more particularly to the method comprising; 1) culturing phytomixotrophic cells and candidates of plant growth regulator which were added in a microwell plate, 2) treating 2,3,5-triphenyltetrazolium chloride thereto, 3) reacting thereof by adding ethanol after removing solutions from microwells, 4) transferring the reacting solution into the new microwell plate, and 5) measuring optical density with a high throughput screening reader. Since the method of the present invention can rapidly and conveniently screen many samples and can also evaluate *in vivo* activities of plant growth regulators, it can effectively be used as a screening method for plant growth inhibitors and activators.

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METHOD FOR HIGH THROUGHPUT SCREENING OF PLANT GROWTH REGULATOR

FIELD OF THE INVENTION

The present invention relates to a method for high throughput screening of plant growth regulators, more particularly to the method comprising the steps of culturing photomixotrophic cells to which candidates of plant growth regulator were added and measuring cell growth on a large scale at the same time.

BACKGROUND

The primary evaluation on the efficacy of a drug, that is a fundamental condition for the development of a plant growth regulator (PGR) including herbicides, has been generally done by investigating the effect of a drug on plant growth by making young plants raised in a greenhouse. The said method is good for the direct investigation of plant growth inhibition but has problems of requiring much time, expense and huge amount of drugs in the early stage of efficacy evaluation.

In the meantime, to develop a medicine, following steps are generally required, that is, investigating

the activity (cytotoxicity) of compounds to human cell lines *in vitro*, applying the compounds having the activity to test animals to detect the toxicity and efficacy of the compounds, determining candidates having excellent activity without toxicity and finally carrying out clinical tests with the candidates (Skehan et al., *J Natl Cancer Inst* 82: 1107, 1990). Likewise, *in vitro* evaluation on the activity of plant growth regulators including herbicides is primarily required for the evaluation of their efficacy. The primary *in vitro* investigation on the activity of herbicides used to be done by using cell-free system taken out of plants. However, the result had no consistency with that of *in vivo* test using plants or if any, it was far from practical use. For instance, even though a PET (photosynthetic electron transport) inhibiting compound having strong activity, confirmed by Hill reaction using thylakoid membrane, the compound did not show any herbicidal activity (Asami et al., *Agric Biol Chem* 51: 205-210, 1987; Sato et al., *Z Naturforsch* 26c: 563-568, 1991).

Plant cell culture techniques including techniques to develop plant transformants using recombinant DNA, to mass-proliferate useful plants by somatic cell culture and to mass-produce the useful

materials by cell culture are the core and fundamental techniques in the field of plant biotechnology. Plant culture cells are comparatively even cell group, which the administered subjects are easily absorbed to and whose culture conditions are regulated freely and accurately. Besides, the efficacy of compounds can be measured with less expense and samples. But most plant culture cells are growing depending upon carbon source supplied from outside because differentiation and development of chloroplasts do not occurring therein. More than half of the conventional herbicides were made to target on chloroplasts including photosynthetic electron transport system. Thus, in order to screen the activity of those herbicides based on plant culture cell system, it is required to use photomixotrophic cells wherein chloroplasts are differentiated well (Dalton, *Biochem Soc Trans* 8: 475-477, 1980; Nishida et al., *Plant Cell Physiol* 21: 47-55, 1980; Sato et al., *Plant Cell Rep* 6: 401-404, 1987). Reports have been made that the herbicidal activity can be investigated using photomixotrophic cells by measuring cell weight, oxygen generation using oxygen electrode and ion conductivity using ion conductance meter (Sato et al., *Z Naturforsch* 26c: 563-568, 1991; Kwon et al., *Kor J Plant Tissue Cult* 26: 183-187, 1999). However, those mentioned measurements are not preferred since all the

measuring takes have to be done by hand (the automation of experiment are not easy) and the experiment scale may not be reduced owing to the matters of measuring methods.

Thus, the present inventors have cultured photomixotrophic cells wherein chloroplasts are differentiated well on a microwell plate along with compounds (synthetic compound, natural compound) or natural extracts (plant extracts, culture solution of microorganism) for a while, and then added reagents generally used for the confirmation of cell viability thereto, followed by automatic measurement of the effect of those compounds on cell growth using high throughput screening reader. The present inventors have completed this invention by developing a novel screening method for plant growth regulators that investigates the activity of plants efficiently by screening large number of compounds or extracts fast and simultaneously even with small amount of samples.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention provides a method for high throughput screening of plant growth regulators using photomixotrophic cells.

Further features of the present invention will appear hereinafter.

The present invention provides a method for high throughput screening of plant growth regulators in which photomixotrophic cells are cultured with candidates of plant growth regulator and then cell growth is measured simultaneously on a large scale.

In order to analyze the activity of abundant compounds to plants simultaneously and efficiently even with small amount of samples, following conditions are required:

1) Establishment of proper culture cells that can reflect the activity to plants;

2) Establishment of culture system for simultaneous screening of abundant compounds even with small amount of samples;

3) Establishment of a simple method for quantitative analysis of the activity; and

4) Establishment of an automatic evaluation system for the activity.

In the present invention, photomixotrophic cells were used as proper culture cells that can reflect the effect of plant growth regulators on plants. Photomixotrophic cells can be selected from a group consisting of *Amaranthus cruentus*, *Asparagus*

officinalis, *Chenopodium rubrum*, *Cytisus scoparius*, *Datura innoxia*, *Digitalis purpurea*, *Glycine max*, *Gossypium hirsutum*, *Hyoscyamus niger*, *Nicotiana tabacum* (tobacco), *Marchantia polymorpha* (liverwort), *Spinachia oleracea* and *Solanum tuberosum* (*Plant Tissue Culture* 3: 147-155. 1986). Among them, *Marchantia polymorpha* or *Nicotiana tabacum* photomixotrophic cells are preferred.

The photomixotrophic cells of the present invention have the same chloroplast structure as higher plants have. The growth speed of those cells is very fast and the cells are uniform, so that they can be inoculated by the fixed concentration and cultured even in a microwell size plates. Plant culture cells are rather a uniform cell group which the administered substances are easily absorbed to and whose culture conditions can be regulated freely and accurately. The plant culture cells are also useful for measuring the efficacy of compounds with small amount of samples and less expense. However, most plant culture cells are heterotroph, that is, carbon source should be supplied from outside because chloroplasts are not differentiated therein. In the present invention, liverwort (*Marchantia polymorpha* L.) (Ohta et al., Planta 136: 229-232, 1977) and tobacco (*Nicotiana tabacum* cv. BY4) photomixotrophic cells (Cha et al., Korean J Bot 36: 113-120, 1993) were used since

chloroplasts were differentiated well in those cells and the cells were uniform and growing fast.

The photomixotrophic cells of the present invention means photomixotrophic cultured cells (PM cells), which are the plant culture cells growing better when carbon source is supplied from outside even though they have differentiated chloroplasts. In addition to the photomixotrophic cultured cells, heterotrophic cultured cells in which chloroplasts are not differentiated or photoautotrophic cultured cells that can grow without carbon source supplied from outside since chloroplasts are differentiated therein might be the candidate for plant culture cells of the present invention. However, they are not suitable for the large scale screening of the present invention since heterotrophic cultured cells do not have differentiated chloroplasts and photoautotrophic cultured cells show very slow growth speed.

In the present invention, photomixotrophic cells were cultured in a microwell plate where candidates of a plant growth regulator were added in order to establish a culture system that makes mass screening with small amount of samples possible. Every microwell plates which have been generally used for cell culture can be used for the present invention and especially,

one selected from a group consisting of 24 microwell plate, 96 microwell plate, 386 microwell plate, 960 microwell plate and 9600 microwell plate is preferably used.

In the age of nano-technology, the primary screening of compounds should give required results even with small amount of samples. In order to investigate the efficacy of plant growth regulators including herbicides, it is preferable to evaluate *in vitro* activity of the regulators first and then carry out *in vivo* tests with compounds confirmed to have the activity using plants. In this invention, plant culture cells were distributed into wells of microwell plate and cultured effectively in medium added into the wells (10 - 1000 $\mu\ell$/well). Even with a very small amount of compounds, it was possible to repeat the experiment several times. Only when the activity is detected at the level of 1 ppm in the primary screening, it proceeds with the secondary screening. Therefore, the culture scale of the present invention using microwell plate seems to be very reasonable.

For the screening of plant growth regulators, every possible substance can be used, and particularly, it is preferable to choose one from a group consisting of a synthetic compound, a pure compound including natural substances, plant extracts and extracts or

fractions containing culture solution of microorganism. As treat candidates for plant growth regulator to plant culture cells, it is possible to treat different candidates at the same time, to treat a candidate with different concentrations or to treat different candidates with different concentrations simultaneously. Just one screening over a microwell plate enables to measure the effect of candidates for plant growth regulator on plant growth, for which different candidates are treated with different concentrations at the same time. The above method of the present invention ensures correct screening without experimental errors caused by individual screening or tests.

In order to establish a simple treatment method for the quantitative analysis on the activity, the present inventors added 2,3,5-triphenyltetrazolium chlorolide (referred as "TTC" hereinafter) to photomixotrophic cultured cells and measured optical density, leading to the measurement of cell growth.

It is not easy in microwell size culture (150 ㎕ /well) to measure ion concentration of a medium or the weight of cultured cells after a given period of time from being treated with compounds since the amount of initial inoculation is under ㎍. In order to

investigate the effect of compounds on cell growth, the present inventors used TTC compound that has been widely used for investigating cell viability and makes quantitative measurement of cell damage extent possible owing to its color reaction. When TTC compound is reacted with an enzyme of mitochondrial inner membrane, it turns into a deep red formazan by reduction (Lakon, Ber Dtsch Bot Ges 60: 299, 1942). Therefore, the undamaged cells by compounds turn into red by the reaction with TTC, but damaged cells loose color. The absorption wavelength of the converted formazan is around 490 nm. Thus, the efficacy of compounds can be evaluated simply by measuring the optical density at 490 nm. If the optical density of a group treated with compounds is lower than that of a compound untreated group, the treated compounds must inhibit plant cell growth. On the contrary, if the optical density of a group treated with compounds is higher than that of a compound untreated group, the compounds must promote plant cell growth. Therefore, the screening method of the present invention can be effectively used not only for the screening of plant growth inhibitors but also for the screening of plant growth promoters.

In order to establish an automatic system to analyze the activity of various kinds of compounds

quantitatively and shortly, the present inventors used high throughput screening (HTS) reader enabling to measure the absorption wavelength of color reaction products simultaneously in a short time. As of today, in order to confirm cell viability, it was general to add solvent to cells and then homogenize, followed by centrifugation. Then, measured optical density at last. But the way was not suitable for quantitative analysis of many samples at the same time. Therefore, in the preferred embodiment of the present invention, added compounds to culture cells and stopped the culture. Then, removed medium with multi-pipette and then added a certain amount of ethanol. After a while, reaction formazan generated by TTC reaction was separated from cells, by which the efficacy of compounds could be effectively evaluated without a troublesome cell-crushing procedure (see FIG. 2).

The method for screening of plant growth regulators of the present invention comprising the following steps:

1) Culturing photomixotrophic cells in a microwell plate to which candidates for a plant growth regulator are added;

2) Treating 2,3,5-triphenyltetrazolium chlorolide thereto;

3) Reacting thereof by adding ethanol after removing solutions from the microwell plate;

4) Transferring the reacting solution of the above step 3) into a new microwell plate; and

5) Measuring optical density of the microwell plate of the above step 4) with a high throughput screening reader.

As for the step 2), the treatment time of 2,3,5-triphenyltetrazolium chlorolide is generally 3-7 hours, but 4.5-5.5 hours are preferable and 5 hour treatment is the most preferable.

As for the step 3) the ethanol is preferably 10-100% ethanol, 85-100% is more preferable and 95% ethanol is the most preferable. After adding ethanol, it is preferable to induce reaction at 70℃ for 0.1-3 hours. It is more preferable to induce reaction at 55-65℃ for 0.5-2 hours and 1 hour reaction at 60℃ is the most preferable.

In the preferred embodiments of the present invention, the present inventors investigated the effect of herbicides as candidates for a plant growth regulator on photomixotrophic cells or heterotrophic cultured cells. As a result, herbicides showed better herbicidal activity in photomixotrophic cells having well-differentiated chloroplasts than in heterotrophic

cultured cells having undifferentiated chloroplasts. When synthetic compounds or natural compounds were treated to photomixotrophic cells even with low concentrations, the activity to plants was clearly detected (see Table 2 and Table 3). The screening system of the present invention is very useful for screening of plant growth regulators with compounds, plant extracts or culture solution of microorganism and for the purification of the activating substances (see Table 4 - Table 7).

As explained hereinbefore, the present inventors established a system suitable for the effective evaluation on large number of compounds using less samples in a short period of time with reflecting their in vitro activity to plants well enough by using photomixotrophic cells. Again, it is possible to screen plant growth regulators with less expense in a short period of time, with the screening method of the present invention. Thus, the method can be effectively used for the development of plant growth regulators such as herbicides or growth promoters.

BRIEF DESCRIPTION OF THE DRAWINGS

The application of the preferred embodiments of the present invention is best understood with

reference to the accompanying drawings, wherein:

FIG. 1 is a set of photographs showing the callus cultures (upper panel) of *Marchantia polymorpha* L., *Nicotiana tabacum* cv. (BY4) and *Oryza sativa* L. cv (Taebaegbyeo) and suspension cultures (lower panel) of those in Erlenmeyer flasks;

FIG. 2 is a set of photographs showing the results of screening of plant growth regulators after subculturing *Marchantia polymorpha* L. cells in a 96 well plate. The upper panel represents the state before treating compounds to culture cells, the middle panel represents the state on the 7th day from treating the compounds and the lower panel represents the state on the 5th hour from treating TTC to the cells of day 7 after treating the compounds;

FIG. 3 is a set of graphs showing the amount of formazan generated in cells and media on the first and fifth hour each after treating TTC to *Marchantia polymorpha* L. cells of day 7 which had been treated with atrazine, a photosynthesis-inhibiting herbicide, by different concentrations (final conc.: 0, 0.1, 0.3, 1, 3, 10, 30 μ M) in 12 well plates.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: Culture of *Marchantia polymorpha* L. and *Nicotiana tabacum* cv. (BY4) photomixotrophic cells having differentiated chloroplasts

The present inventors used cells developed by Ohta et al. (Ohta *et al.*, *Planta*, 136: 229-232, 1977) as *Marchantia polymorpha* L. photomixotrophic cells. Medium was prepared by using M51 medium (Furner *et al.*, *Plant Sci Lett*, 11: 169-176, 1978) for vitamins and macronutrients, and B5 medium (Gamborg *et al.*, *Exp Cell Res*, 50: 151-158, 1968) for casamino acid, glutamine and micronutrients including 2,4-D (Table 1). Suspension cultured *Marchantia polymorpha* L. cells were inoculated by 0.5 g into a 250 ㎖ flask containing 50 ㎖ of liquid medium prepared as the composition of Table 1.

Then, the cells were suspension cultured at 25℃, 100 rpm under 15 μ mol $m^{-2} \cdot s^{-1}$ light condition (FIG. 1). The cells were sub-cultured at intervals of 9 days.

<Table 1>

The medium composition used for photomixotrophic cell culture.

Macronutrients (/ℓ)		Micronutrients (/ℓ)		Vitamins (/ℓ)		Others (/ℓ)	
NH_4NO_3	400 mg	KI	0.75 mg	Myo-inositol	100 mg	Casamino acid	1 g
KNO_3	2 g	H_3BO_3	3 mg	Thiamine-HCl	10 mg	L-glutamine	200 mg
$CaCl_2$	300 mg	$MnSO_4 \cdot 4H_2O$	10 mg	Nicotinic acid	1 mg	2,4-D	1 mg
KH_4PO_2	275 mg	$ZnSO_4 \cdot 7H_2O$	2 mg	Pyridoxine-HCl	1 mg	Sucrose	20 g
$MgSO_4 \cdot 7H_2O$	370 mg	$CuSO_4 \cdot 5H_2O$	0.025 mg	$FeSO_4 \cdot 7H_2O$	27.9 mg		
		$CoCl_2 \cdot 6H_2O$	0.025 mg	Na_2-EDTA	37.3 mg		
		$Na_2MoO_4 \cdot 2H_2O$	0.25 mg				

As *Nicotiana tabacum* cv. (BY4) photomixotrophic cells, the cells developed as NaCl resistance cells by Cha et al. (Cha *et al.*, *Korean J Bot*, 36: 113-120, 1993) were used. For the culture of the above cells, MS minimal medium where 0.7 mg/ℓ of 2,4-D and 0.03 mg/

ℓ of kinetin were added was used. 2 g of *Nicotiana tabacum* cv. (BY4) photomixotrophic cells was inoculated into a 250 mℓ flask containing 50 mℓ of liquid medium, and then suspension cultured at 25℃, 100 rpm under 15 μ mol $m^{-2} \cdot s^{-1}$ light condition (FIG. 1). The cells were sub-cultured at intervals of 14 days.

For the preparation of non-photomixotrophic cells (*Oryza sativa* L. cv Taebaegbyeo), cultured cells induced from an immature embryo (Jeong *et al.*, *Korean J Plant Tissue Culture*, 18: 209-214, 1991) in N6 liquid medium where 2,4-D was added by 1 mg/ℓ . Other culture conditions were same as those for *Marchantia polymorpha* L. photomixotrophic cell culture.

Example 2: Analysis on the effect of photosynthesis inhibiting herbicides after treating TTC during cell culture in microwells

Every compounds and extracts used in this invention were dissolved in acetone, N,N-dimethylformamide (DMF), etc and then treated into each well by 1.5 μℓ aseptically. The final concentration of organic solvents used for dissolving each compound was adjusted to 1%(1.5 μℓ/150 μℓ).

The *Marchantia polymorpha* L. photomixotrophic

cells sub-cultured in the above Example 1 were suspended in 200 ㎖ of liquid medium by 3 g of biomass on the 2nd - 3rd day of subculture and then distributed into each well of a 96 microwell plate by 150 ㎕ at the concentration of 0.2 ㎍ biomass/150 ㎕/well (FIG. 2). In order to confirm whether the screening method of the present invention worked well, investigated cell viability by treating atrazine, which is a photosynthesis inhibiting herbicide working only in the presence of chloroplast, by 0, 0.1, 0.3, 1, 3, 10 and 30 μ M each on the 7th day of culture. And *Marchantia polymorpha* L. photomixotrophic cells were cultured upto be 100 ㎖ in a flask, which were used for the evaluation of the efficacy of the compounds of the present invention.

In order to investigate cell viability on the 7th day of atrazine treating culture, treated 2,3,5-triphenyl tetrazolium chlorolide (TTC) at the concentration of 12 mM. TTC compound was reacting with an enzyme of live mitochondrial inner membrane (TTC reduction), resulted in the diversion into deep red formazan (Lakon, *Ber Dtsch Bot Ges*, 60: 299, 1942). Therefore, undamaged cells by the treatment of the compounds turned into red color by TTC reaction, but damaged cells had no colors.

The investigation procedure of cell viability in

a 96 microwell plate of the present invention is precisely explained as follows. 1) Treating 150 ㎕ of TTC solution into each well wherein cells are being cultured. 2) Five hours after treating TTC, removing the solution in wells with a 8-channel multi-pipette. 3) Adding 150 ㎕ of 95% ethanol to the remaining cells of each well, and then reacting thereof at 60℃ for 1 hour. 4) After reaction finished, transferring the reacting solution into a new 96 well plate. 5) Measuring the optical density of the well plate at 490 nm wave length with a high throughput screening (HTS) reader. The explained method of the present invention is called high throughput screening.

Before measuring the efficacy of the screening method using a 96 well plate, the present inventors performed TTC analysis using a 12 well plate following the same procedures as the explained high throughput screening method. Again, cultured cells in a 12 well plate where the culture solution of each well was adjusted to 1.5 ㎖. Treated atrazine, a photosynthesis inhibiting herbicide, by different concentrations on the 7^{th} day of culture, after which measured the amount of intracellular or extracellular formazan on the 1^{st} or 5^{th} hour from the treatment. In order to measure the amount of intracellular formazan, treated TTC first and then added 95% ethanol to the remaining cells of each

well, followed by the reaction at 60℃ for an hour. Then, measured the optical density at 490 nm. In order to measure the amount of extracellular formazan, also treated TTC and then collected culture solution to measure the optical density at 490 nm. As a result, after 1 hour from the treatment of TTC, formazan in medium (extracellular formazan) was hardly detected and the content of intracellular formazan was decreased dose-dependently. After 5 hours from the treatment of TTC, both extracellular and intracellular formazan content were decreased dose-dependently. About 50% of formazan were isolated from cells, making the intracellular and extracellular formazan content almost even (FIG. 3). Thus, when TTC was treated for 5 hours, the change of intracellular formazan content was more clearly detected, which seemed to be alike with the growth inhibition curve resulted from the measurement in flask culture.

The present inventors also performed TTC analysis using a 96 well plate by the same procedure as using a 12 well plate. As a result, formazan was not separated enough from the cells 5 hours after the treatment of TTC. But, formazan content could be detected when OD of reacting solution was measured after adding 95% ethanol and further induced reaction at 60℃ for 1 hour, 5 hours after the treatment of TTC.

Every experiment using a 96 well plate included the steps of treating TTC solution for 5 hours, adding 95% ethanol thereto, reacting at 60℃ for an hour and measuring the amount of formazan generated in live cells, which offered an advantage for evaluating the efficacy of compounds easily and shortly, though with large numbers of the compounds. In the experiment using a 96 well plate, atrazine content that can inhibit cell growth upto about 50% was 0.68 μM (0.18 ppm), which was almost the same value as the cell growth inhibiting activity observed in mass-culture of cells in flasks. Thus, the inhibiting activity of compounds induced by TTC reaction reflected the cell growth inhibition pretty well.

Example 3: Evaluation on the efficacy of synthetic compounds

The present inventors investigated the growth inhibiting activity of synthetic herbicides to *Marchantia polymorpha* L. photomixotrophic cells, *Nicotiana tabacum* cv. (BY4) photomixotrophic cells and *Oryza sativa* L. cv (Taebaegbyeo) heterotrophic cells using TTC analysis as performed in the above Example 2 after treating 13 kinds of conventional herbicides

having different reacting mechanisms with different concentrations as stated in Table 2. *Oryza sativa* L. cv (Taebaegbyeo) heterotrophic cells used in this experiment were developed from premature embryo (*Korean J Plant Tissue Culture*, 18: 209-214, 1991).

<Table 2>

Comparison of growth inhibition activities (IC_{50}: ppm) of synthetic herbicides to *Marchantia polymorpha* L. photomixotrophic cells, *Nicotiana tabacum* cv. (BY4) photomixotrophic cells and *Oryza sativa* L. cv (Taebaegbyeo) heterotrophic cells.

Herbicide	*Marchantia polymorpha* L. photomixotrophic cells	*Nicotiana tabacum* cv. (BY4) photomixotrophic cells	*Oryza sativa* L. cv (Taebaegbyeo) heterotrophic cells	Reaction mechanism (Inhibition target))
Atrazine	0.21	0.18	>10	Photosynthetic electron transport system
Linurone	0.244	>10	7.07	Photosynthetic electron transport system
Propanil	0.037	5.83	8.49	Photosynthetic electron transport system
Chloromethoxynil	0.076	0.193	>10	Protoporphyrin IX

Oxadiazone	0.03	0.39	6.88	Protoporphyrin IX
Diflufenican	0.031	4.67	>10	Carotenoid synthesis
Dithiopyr	0.048	0.141	0.613	Lipid synthesis
Phenoxapro-P-ethyl	0.70	4.58	6.47	ACCase synthesis
Amazapyr	0.35	0.018	0.269	Acetolactate synthase Synthesis
LGC-42153	0.0011	0.0022	1.793	Acetolactate synthase Synthesis
Prazolsulfuron-ethyl	0.0012	0.001	0.066	Acetolactate synthase Synthesis
Pyribenzoxim	0.0092	0.0026	7.83	Acetolactate synthase Synthesis
Naproanilide	0.054	0.0019	6.69	Acetolactate synthase Synthesis

In the above table, ACCase is 1-aminocyclopropane-1-carboxylic acid synthase.

As a result, LGC-42153, amazapyr, prazolsulfuron-ethyl, pyribenzoxim and naproanilide which are the herbicides having acetolactate synthase (ALS) inhibiting activity showed high growth inhibiting activity even with small amount in photomixotrophic cells. Especially herbicides targeting chloroplasts were proved to have strong growth inhibiting activity to photomixotrophic cells and following herbicides ought to be included in that category; 1) atrazine,

linurone and propanil which inhibit photosynthetic II (PSII), 2) chloromethoxynil and oxadiazone which inhibit synthesis of protoporphyrin IX (protox), an intermediate of chlorophyll, 3) diflufenican which inhibits carotenoid synthesis. Among those herbicides, atrazine, chloromethoxynil and diflufenican showed low growth inhibiting activity or almost none even with 10 ppm to non-photomixotrophic (heterotrophic) cells (Table 2). The effects of herbicides on the market on culture cells were various. And plant growth inhibiting activities of those were better detected in photomixotrophic cells having well-differentiated chloroplasts, comparing with heterotrophic cells whose chloroplasts were not differentiated. Therefore, the method to measure formazan generated by TTC reaction with HTS reader, after culturing photomixotrophic cells in a 96 well plate, was confirmed to be very effective to measure weeding activity of the compounds.

Example 4: Evaluation on the efficacy of natural compounds

In order to investigate the growth inhibiting activity to *Marchantia polymorpha* L. photomixotrophic cells of natural compounds isolated from various plants

(Table 3), the present inventors performed the efficacy evaluation test used in the above Example 2, and then compared the results with the growth inhibiting activity to *Lemna paucicostata*, an aquatic plant (Korea Research Institute of Chemical Technology). Adjusted the final concentrations of the compounds treated to *Marchantia polymorpha* L. photomixotrophic cells to 1 ppm and adjusted the final concentrations of the compounds treated to *Lemna paucicostata* to 31 ppm.

Measured the growth inhibiting activity of the compounds to *Lemna paucicostata* as follows; Cultured *Lemna paucicostata* in a 24 well plate containing Hutner's nutrient medium, which was treated with 16 different natural compounds stated in Table 3. Investigated the growth inhibiting activity 5 days after treating. For the evaluation of the inhibiting activity, classified the grades into 6 from 0 to 5 based on the naked eye distinction. Grade 0 was defined as having under 10% inhibiting activity, so was grade 1 as having 11-30% activity, grade 3 as having 51-70% activity, grade 4 as having 71-90% activity, and grade 5 as having 91-100% inhibiting activity. Investigated the growth inhibiting activities of the compounds to *Marchantia polymorpha* L. photomixotrophic cells using the same method as in Example 2. Calculated the inhibiting activity (% inhibiting

activity) by 1-(OD of compound-treated group/OD of control) X 100. + value means the cell growth inhibiting activity and - value means the cell growth promoting activity.

As a result, the 16 different compounds used in this invention were confirmed to have growth inhibiting activity to *Marchantia polymorpha* L. photomixotrophic cells though it varied, among which coumarine showed the strongest growth inhibiting activity (33%). The growth inhibiting activity to *Marchantia polymorpha* L. photomixotrophic cells was in proportion to the activity to *Lemna paucicostata*, one of aquatic plant. Interestingly, ferulic acid, dicumarol and 3-coumaranone were proved to have the growth promoting activity to *Marchantia polymorpha* L. photomixotrophic cells (Table 3). *Marchantia polymorpha* L. photomixotrophic cells were easily affected by natural compounds even with low concentration, meaning that small amount of treated natural compounds could inhibit the cell growth, making them a useful candidate for the method for screening of plant growth regulators of the present invention.

<Table 3>

Growth inhibition activities of 16 compounds isolated from plants to *Marchantia polymorpha* L.

photomixotrophic cells and *Lemna paucicostata*.

Compounds	*Marchantia polymorpha* L. photomixotrophic cells (% activity)	*Lemna paucicostata* (% activity)
Benzoic acid	9	10
Caffeic acid	21	30
Coumarin	33	80
Dicumarol	-31	30
o-Coumaric acid	32	30
p-Coumaric acid	17	30
3-Coumaranone	-18	0
Ferulic acid	-35	20
Gallic acid	8	0
Gentistic acid	9	0
Hydroquinone	15	0
Protocatechuic acid ethyl ester	22	70
Scopoletin	16	0
Syringic acid	16	0
Umbelliferone	6	40
Vanillic acid	10	10

Example 5: Evaluation on the efficacy of plant extracts (fractions)

The present inventors investigated the effect of methanol extracts (final conc.: 10 ppm) extracted from 49 kinds of plants including fruits of *Viburnum*

dilatatum provided by Korea Plant Extract Bank of Plant Diversity Research Center on *Marchantia polymorpha* L. photomixotrophic cells with the same method used in the above Example 2.

As a result, the effects of those plant extracts on plant cell growth were varied. The extracts of *Daphniphylium macropodum* small branch, *Ribes fasclculatum* var. Chinese fruit, *Valeriana officinalis* var. latifolia leaf and trunk/root and *Trichosanthes kinilowii* var. japonica seed were confirmed to have more than 50% growth inhibiting activity to *Marchantia polymorpha* L. photomixotrophic cells (Table 4 and Table 5).

<Table 4>

The effect of 49 kinds of plant extracts on the growth of *Marchantia polymorpha* L. photomixotrophic cells.

Plant name (Scientific name)	Region	OD_{490}	Inhibition activity (%)
Control		0.808	0
Viburnum dilatatum	Fruit	0.536	34
Ilex integra	Leaf	0.735	9
Ilex integra	Trunk-heartwood	0.680	16

Ilex integra	Trunk-bark	0.636	21
Quercus gilva	Leaf	0.756	6
Quercus gilva	Trunk-heartwood	0.735	9
Quercus gilva	Trunk-bark	0.841	0
Cayratia japonica	Leaf	0.802	1
Cayratia japonica	Fruit	0.623	23
Celtis choseniana	Leaf	0.560	31
Celtis choseniana	Trunk	0.570	29
Celtis choseniana	Fruit	0.668	17
Staphylea bumalda	Fruit	0.695	14
Staphylea bumalda	Pericarp	0.594	26
Wasabia koreana	Root	0.671	17
Ligustrum japonicum	Leaf	0.758	6
Ligustrum japonicum	Small branch	0.619	23
Castanopsis cuspidata var. *sieboldii*	Leaf	0.564	30
Castanopsis cuspidata var. *sieboldii*	Trunk-heartwood	0.530	34
Castanopsis cuspidata var. *sieboldii*	Trunk-bark	0.708	12
Dephniphyllum macropodum	Leaf	0.602	25
Dephniphyllum macropodum	Trunk	0.619	23
Dephniphyllum macropodum	Fruit	0.692	14
Dephniphyllum macropodum	Leaf	0.445	45
Dephniphyllum macropodum	Small branch	0.380	53

<Table 5>

The effect of 49 kinds of plant extracts on the growth

of *Marchantia polymorpha* L. photomixotrophic cells.

Plant name (Scientific name)	Region	OD_{490}	Inhibition activity (%)
Ribes fasciculatum var. *chinense*	Fruit	0.356	56
Ribes fasciculatum var. *chinense*	Trunk	0.428	47
Litsea japonica	Leaf	0.642	21
Litsea japonica	Trunk-heartwood	0.499	38
Litsea japonica	Trunk-bark	0.573	29
Catalpa bignonioides	Fruit	0.409	49
Valeriana officinallis var. *latifolia*	Leaf	0.371	54
Valeriana officinallis var. *latifolia*	Trunk, root	0.364	55
Trichosanthes kinilowii var. *jponica*	Seed	0.379	53
Trichosanthes kinilowii var. *jponica*	Sarcocarp	0.409	49
Cinnamomum camphora	Trunk-heartwood	0.484	40
Cinnamomum camphora	Trunk-bark	0.620	23
Clerodendrum trichotomum	Leaf	0.481	40
Clerodendrum trichotomum	Trunk	0.635	21
Carpesium abrotanoides	Leaf	0.644	20
Carpesium abrotanoides	Trunk	0.749	7
Carpesium abrotanoides	Root	0.765	5
Elaeocarpus sylvestris var. *ellipticus*	Leaf	0.657	19

Cocculus trilobus	Leaf	0.639	21
Cocculus trilobus	Fruit	0.628	22
Viburmum erosum	Fruit	0.668	17
Aralia continentalis	Leaf	0.696	14
Aralia continentalis	Trunk	0.672	17
Aralia continentalis	Fruit	0.626	23

As seen hereinbefore, the photomixotrophic cells were available for the investigation of the activities of synthetic or natural compounds and of plant extracts as well. Therefore, it is highly expected that the HTS system of the present invention based on the use of photomixotrophic cells, can be effectively used for screening plant growth regulators and activators.

Example 6: Evaluation on the efficacy of microorganism culture solution

The present inventors investigated the growth inhibiting activity of culture solution of *Actinomycetes* spp. isolated from soil with the method used in Example 2. Then, the results were compared with results of pot test using *Lemna paucicostata* and other weeds. *Actinomycetes* spp. used in this invention was identified after thorough examination of

microorganisms separated from soils everywhere in Korea and was named as stated in Table 6 and Table 7. The autoclaved culture solution used here was prepared by autoclaving the culture solution at 121℃ for 5 hours and treated with 1 ppm. In order to prepare ethyl acetate (EtOAc) extracts from culture solution, mixed 1 mℓ of culture solution and 1 mℓ of ethyl acetate, and then centrifuged. Removed the lower layer (water layer), concentrated the obtained ethyl acetate layer and treated the concentrated ethyl acetate with 10 ppm. In order to investigate the growth inhibiting activity of the microorganism culture solution to *Lemna paucicostata*, treated the unsterilized microorganism culture solution thereto by 31 ppm. The test procedure was same as performed in Example 4. And for the pot test in a green house, sowed *Abutilon avicennae*Velvetleaf, *Aeschynomene indica*, *Agropyron smithii*, *Calystegia japonica*, *Digitaria sanguinalis*, *Echinochloa crus-galli*, *Monochoria vaginalis*, *Oryzae sativa*, *Panicum dichotomiflorum*, *Sagittaria pygmaea*, *Scirpus juncoides*, *Solanum nigrum*, *Sorghum bicolor*, *Trifolium repens* and *Xanthium strumarium* in test pots (350 cm^2), and cultivated according to the conventional method. Treated the unsterilized microorganism culture solution (0.1% tween 20 solution, final concentration was adjusted to 40 kg/ha) to the leaves of all the

above mentioned plants using a hand sprayer. Cultivated them in a green house for 2 weeks, after which performed pot test. The weeding activity was classified into 6 grades by the pot test based on the naked eye investigation on the morphological and physiological aspects. That is, grade 0 suggests that the inhibiting activity is under 10%, likewise, grade 1 means 20-30% inhibiting activity, grade 2 suggests 40-50% inhibiting activity, grade 3 suggests 60-70% inhibiting activity, grade 4 suggests 80-90% inhibiting activity and grade 5 suggests 100% complete inhibiting activity. The classification was made by the weeding activity to at least a kind of plant. The inhibiting activity value was obtained by averaging the inhibiting activities to all the plants.

As a result, when *Marchantia polymorpha* L. photomixotrophic cells were treated with 1 ppm of autoclaved microorganism culture solutions, two strains (M531 and M774) showed 91% inhibiting activity and other 14 strains showed over 30% inhibiting activity. When *Marchantia polymorpha* L. photomixotrophic cells were treated with 10 ppm of ethyl acetate extracts, 9 strains (G715, G747, G774, G793, M690, M715, M755, M774) showed over 90% inhibiting activity and other 31 strains did more than 30% inhibiting activity. But

interestingly, the autoclaved culture solutions of 32 strains including M715, M912, G745, M755, M281, etc. rather promoted cell growth, making those strains as useful candidates for plant growth stimulators. The growth inhibiting activity to *Marchantia polymorpha* L. photomixotrophic cells was closely related with the results of pot test with *Lemna paucicostata* and plants. That is, a compound confirmed to have inhibiting activity by pot test showed similar activity when used in *Marchantia polymorpha* L. photomixotrophic cells. Culture solutions of 42 strains did not have inhibiting activity to any of *Marchantia polymorpha* L. photomixotrophic cells, *Lemna paucicostata* and plants (Table 6 and Table 7). Therefore, the HTS system using photomixotrophic cells developed by the present inventors has been proved to be a very useful method for screening plant growth regulators in pure compounds, plant extracts, microorganism culture solutions and culture extracts.

<Table 6>

The activity of soil *Actinomycetes* spp. culture solution to *Marchantia polymorpha* L. photomixotrophic cells, *Lemna paucicostata* and pot test.

Strain	*Marchantia polymorpha* L. photomixotrophic cells		*Lemna paucicostata*	Pot test
	Autoclaved culture solution	Ethyl acetate extracts		
G247	-28	18	0	0
G280	45	85	0	0
G285	32	63	5	5
G297	-47	2	0	0
G325	-5	-12	3	0
G326	-31	-9	0	0
G360	-13	33	0	0
G370	0	0	1	0
G373	13	35	3	0
G408	-2	24	0	3
G410	23	51	2	0
G411	-17	57	0	0
G450	-42	40	0	4
G451	-35	2	0	4
G542	24	-30	0	0
G615	-11	46	0	5
G652	32	55	0	0
G669	40	-17	5	0
G715	45	96	5	3
G719	-181	-4	3	0
G745	-148	-14	4	0
G747	47	99	0	4
G765	-7	-8	4	0
G774	-49	92	4	0
G793	-13	90	0	0
G860	-35	2	4	0

G883	49	-3	4	0
G1160	25	47	4	0
G1175	21	30	4	0

<Table 7>

The activity of soil *Actinomycetes* spp. culture solution to *Marchantia polymorpha* L. photomixotrophic cells, *Lemna paucicostata* and pot test.

Strain	*Marchantia polymorpha* L. photomixotrophic cells		*Lemna paucicostata*	Pot test
	Autoclaved culture solution	Ethyl acetate extracts		
M252	-50	77	4	0
M253	7	-34	4	0
M261	32	46	0	0
M281	-133	93	0	0
M360	-68	53	0	0
M366	-22	30	0	0
M407	-45	9	0	0
M413	21	15	3	0
M443	-101	-2	1	0
M447	-31	14	3	5
M453	-39	36	2	0
M531	91	76	0	0
M537	39	58	0	5
M533	9	2	0	0
M621	22	14	0	0
M635	38	-13	4	0

M656	-11	49	0	5
M690	-9	94	4	5
M702	4	-12	0	0
M705	50	52	4	4
M715	-184	97	5	5
M745	-129	-84	4	0
M752	8	14	0	3
M755	-146	96	0	5
M774	91	99	4	5
M787	-65	32	0	0
M912	-148	-12	4	0
M938	-81	92	5	0
M1370	61	42	0	5

In the above Table 6 and Table 7,

% activity of *Marchantia polymorpha* L. = [1 - (OD of compound treated group/OD of control)] X 100,

+ value means that the cell growth inhibiting activity is detected, and - value means that the cell growth promoting activity is detected.

INDUSTRIAL APPLICABILITY

As explained hereinbefore, the screening method of the present invention comprising the steps of culturing photomixotrophic cells having well differentiated chloroplasts in a microwell plate to

which synthetic compounds, natural compounds or natural extracts are added, adding reagents thereto and measuring the cell growth using high throughput screening reader, can offer a great advantage for the evaluation of the efficacy of the compounds by analyzing various compounds shortly and easily even with small amount of the compounds. Therefore, the high throughput screening method of the present invention can be effectively used for the screening and the development of plant growth regulators with less expense in a short period of time.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

What is claimed is

1. A method for high throughput screening of plant growth regulators comprising the steps of culturing photomixotrophic cells to which candidates for plant growth regulators were added and measuring cell growth on a large scale at the same time.

2. The method as set forth in claim 1, wherein the photomixotrophic cells are *Marchantia polymorpha* L. photomixotrophic cells or *Nicotiana tabacum* cv. BY4 photomixotrophic cells.

3. The method as set forth in claim 1, wherein the candidates for plant growth regulators are selected from a group consisting of synthetic compounds, natural compounds, plant extracts and fractions or extracts containing microorganism culture solutions.

4. The method as set forth in claim 1, wherein the culture is carried out in microwell plates.

5. The method as set forth in claim 1, wherein the cell growth measurement is carried out by measuring optical density after treating 2,3,5-

triphenyltetrazolium chlorolide to culture cells.

6. The method as set forth in claim 1, wherein the method comprises the following steps:

1) Culturing photomixotrophic cells in a microwell plate to which candidates for plant growth regulators are added;

2) Treating 2,3,5-triphenyltetrazolium chlorolide thereto;

3) Reacting thereof by adding ethanol after removing solutions from the microwell plate;

4) Transferring the reacting solution of the above step 3) into a new microwell plate; and

5) Measuring optical density of the microwell plate of the above step 4) with a high throughput screening reader.

7. The method as set forth in claim 6, wherein the step 3 is carried out by treating 2,3,5-triphenyltetrazolium chlorolide for 4.5-5.5 hours, removing solutions from microwells, adding 95% ethanol thereto, and then reacting thereof at 60℃ for 1 hour.

FIGURES

FIG. 1

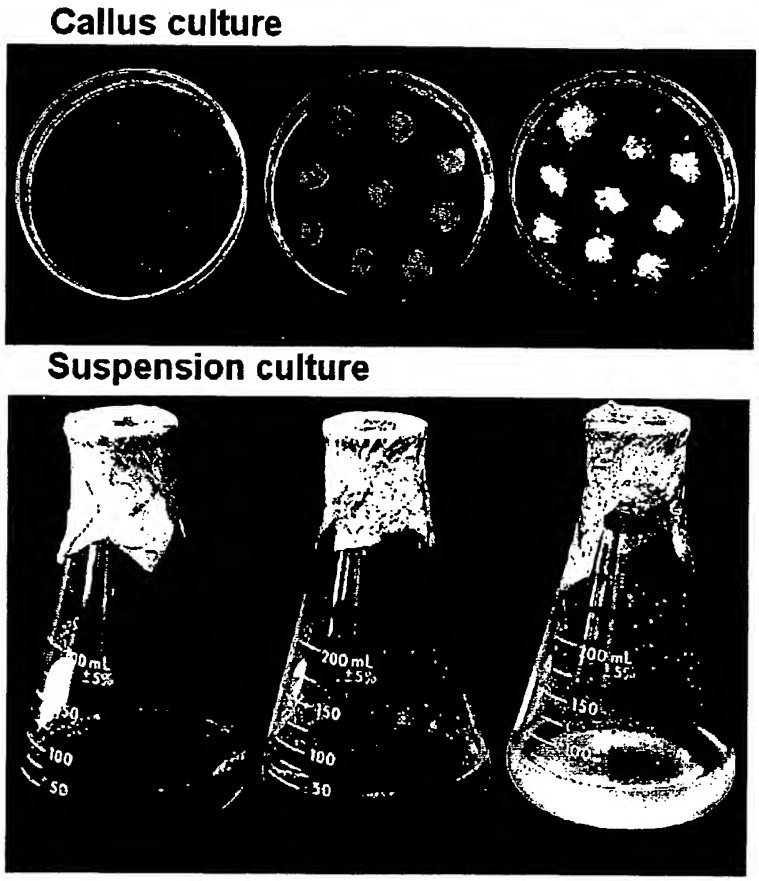


FIG. 2

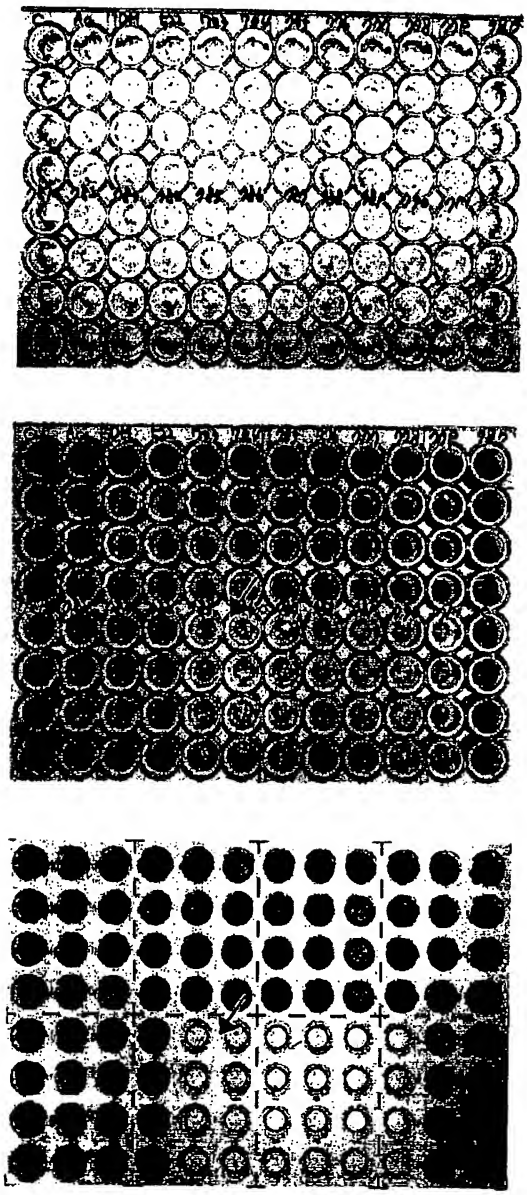
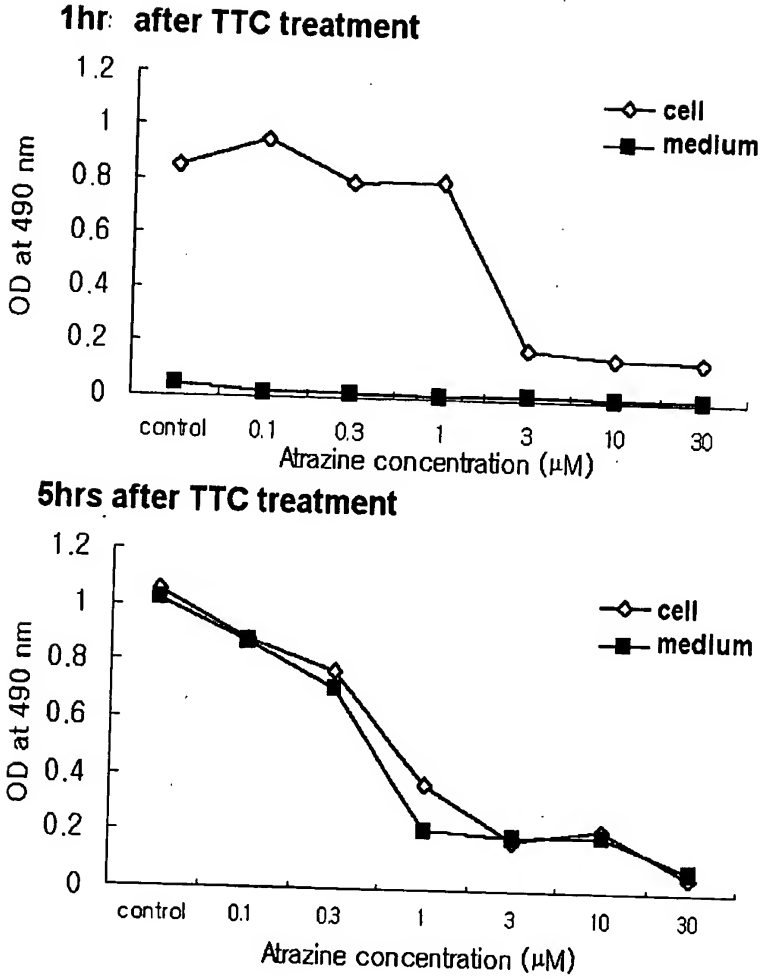


FIG. 3


1hr after TTC treatment
OD at 490 nm
1.2
1
0.8
0.6
0.4
0.2
0
cell
medium
control
0.1
0.3
1
3
10
30
Atrazine concentration (μM)
5hrs after TTC treatment
OD at 490 nm
1.2
1
0.8
0.6
0.4
0.2
0
cell
medium
control
0.1
0.3
1
3
10
30
Atrazine concentration (μM)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR03/01041

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12Q 1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC7 : C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA On-Line

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Sato F. et al. "A comparison of effects of several herbicides on photoautotrophic, photomixotrophic and heterotrophic cultured tobacco cells and seedlings." In: Plant Cell Rep., 1987, 6(6): pages 401-404, see entire document.	1-7
A	Dalton C. "The effect of carbohydrates on the greening of plant cultures." In: Biochem. Soc. Trans., 1980, 8(4): pages 475-477, see entire document.	1-7
A	Rich P.R. et al. "The sites of interaction of triphenyltetrazolium chloride with mitochondrial respiratory chains." In: FEMS Microbiol. Lett., 2001, 202(2): pages 181-187, see entire document.	5-7
A	Otero A.J. et al. "2,3,5-Triphenyl tetrazolium chloride (TTC) reduction as exponential growth phase marker for mammalian cells in culture and for myeloma hybridization experiments." In: Cytotechnology, 1991, 6(2): pages 137-142, see entire document.	5-7

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 SEPTEMBER 2003 (25.09.2003)	26 SEPTEMBER 2003 (26.09.2003)
Name and mailing address of the ISA/KR Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer HAN, Hyung Mee Telephone No. 82-42-481-5601

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